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14. ABSTRACT

Genetic and epigenetic inactivation of *SMAD4* are rare occurrences in breast tumors despite it is localized to chromosome 18q and serves as a frequent target for inactivation in advanced gastrointestinal cancers. On the other hand, our studies demonstrated that *SMAD8* could be an alternate target gene which undergoes epigenetic silencing of gene expression in nearly 30% of breast cancers. These studies provided the first line of evidence for an alternate mechanism for disruption of the Smad signaling events in breast cancer. Smad8 is an R-Smad involved in the regulation of BMP-responsive genes including those affect bone metabolism. Since bone metastasis is frequently associated with breast cancer, it is likely that Smad8 inactivation in breast cancer could play a role in metastasis/ bone metastasis. In the future, we are planning to follow up these critical observations and use model cell lines and mouse models to identify and characterize the mediator and effector genes that regulate metastatic progression of breast cancer due to Smad8 inactivation.

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Table of Contents

FINAL REPORT OF THE USAMRMC FUNDED ACTIVITY

Title of the grant: Metastatic progression of breast cancer by allelic loss on chromosome 18q21.

1. Introduction/ Project Overview/ Scientific Progress and future directions:

An association has been established between the high frequency of deletion of chromosome 18q21, where the *SMAD4* gene is localized, with advanced stages of gastrointestinal cancers (1). These observations received added credence from several subsequent reports which reported an association between increase in the frequency of *SMAD4* mutations and advanced stages of gastrointestinal cancers (2). We set out to test the possibility that a similar situation may exist in breast cancer. However, our experimental data suggested that inactivation of Smad signaling in breast cancer is primarily due to loss of expression of Smad8 rather than due to mutational inactivations of the *SMAD* genes such as *SMAD4* or *SMAD2* localized to chromosome 18q21. Furthermore, our studies also provided the first direct evidence that loss of expression of *SMAD8* was mediated by epigenetic promoter DNA methylation silencing of the gene. Based on our data, we propose that Smad signaling involving Smad8 is an important pathway in the breast tissue and inactivation or loss of Smad8 is a critical tissue specific event in breast tumorigenesis.

Smad8 is an R-Smad that becomes phosphorylated during BMP signaling events and modulates BMP-responsive genes including those that may affect bone metabolism (3). Bone metastases frequently develop in breast cancer patients and the bulk of their tumor burden at the time of their death appears to be in the bone (4). Therefore, in the future, we plan to undertake further studies to understand the role of defective Smad8 signaling in altering differential gene expression causing metastatic conversion in breast cancer. In the long term, we plan to delineate whether defective Smad8 signaling in metastatic breast cancer cells could be responsible for causing an imbalance in the normal bone homeostasis by enhancing osteoclastic activity and/or reduced osteoblast activity leading to osteolytic lesions resulting in bone damage and pain in cancer patients.

2. Results and Discussion:

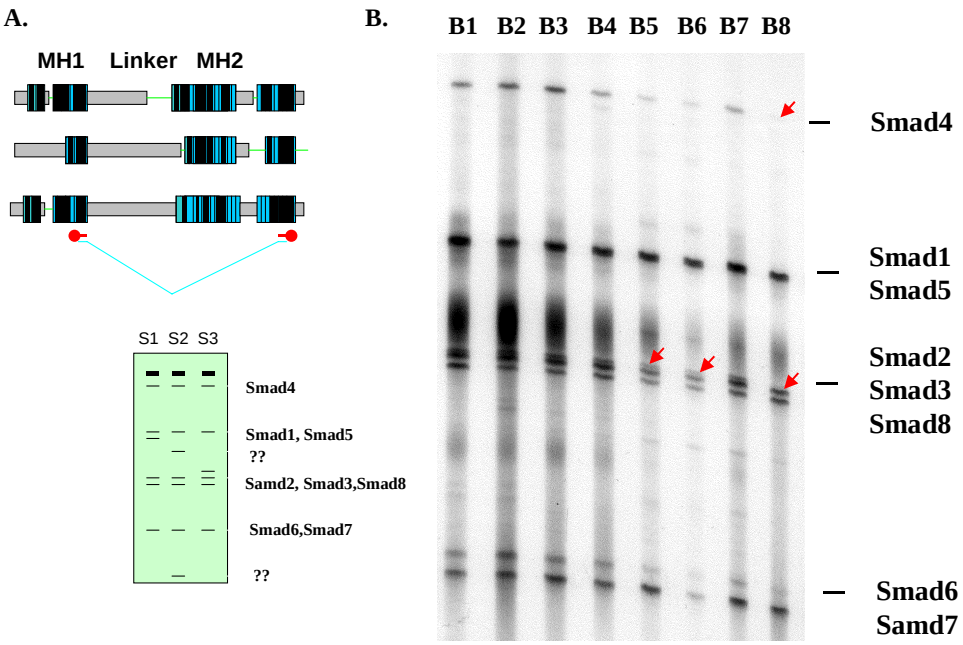

Figure 1. Targeted expressed gene display (TEGD).
A. Schematic representation of TEGD for the SMAD family of genes. MH1 and MH2 indicate highly homologous regions in the aminoacid as well as DNA sequence among the various SMAD gene family members. The forward and reverse primers for PCR amplification of the cDNA were designed in the conserved regions as indicated. The

radiolabeled PCR products were analyzed by denaturing acrylamide gel electrophoresis. B. PCR products for SMADs using degenerate primers were analyzed by TEGD. Lanes B1-8 correspond to PCR products generated using cDNA templates from the normal mammary gland cells (B1) and tumor or cell line (B2-8) samples. B8 is a cell line (MDAMB468) with a homozygous deletion for Smad4 and serves as an internal control. The arrows point to distinct PCR products that were abnormal compared to the normal control. The positions of various SMAD genes and their variants as identified from sequence analysis are indicated on the right panel.

The *SMAD* family of genes encode highly homologous amino acid sequences at their N- and C-terminal regions (MH1 and MH2 respectively), which are separated by a highly divergent linker region (1,5). We have developed a novel technique known as TEGD (targeted expression gene display) based on degenerate PCR using primers corresponding to the conserved regions to simultaneously analyze the *SMAD* family of genes for high throughput routine analysis of their expression patterns (Figure 1). These results indicated to us that TEGD could be used as a tool for initial diagnostic high throughput evaluations to determine *SMAD* gene expression patterns simultaneously with an increased level of sensitivity in cancer. The results from these analyses provided the initial indications that the *SMAD8* gene is a critical target for loss of function due to down regulation of gene expression in various cancers. Subsequent analysis of the *SMAD8* gene using gene specific primers by semi quantitative RT-PCR to confirm the TEGD data exhibited loss of expression in nearly 31% (11/35) of breast cancers (Figure 2).

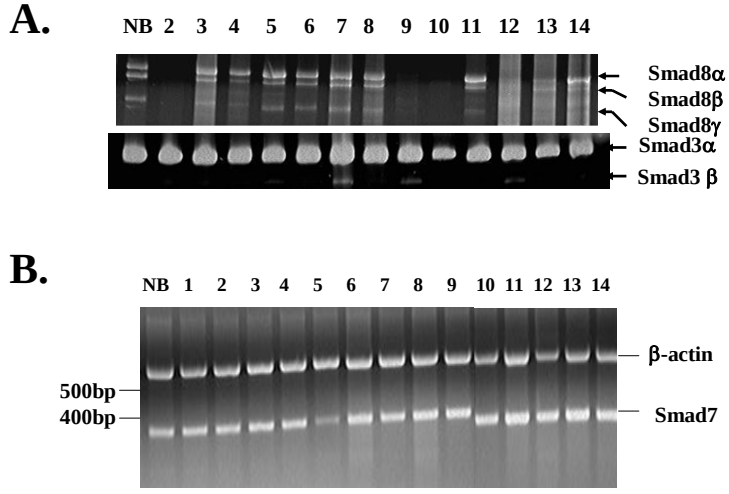

Figure 2. Semi-quantitative RT-PCR analysis of Smad8 expression in breast cancer cell lines and tumors.

A. Total RNA was prepared using the Trizol method from the indicated breast cancer specimens and analyzed by RT-PCR. Lanes 1-4 and 12-14 are primary tumor samples, 5-11 are cell lines. SMAD8α, SMAD8β and SMAD8γ are three of the major differentially spliced forms of Smad8 which correspond to the full-length, deletion of exon 2, and deletions of exons 2&3, respectively. Analysis of the SMAD3 gene is used for normalization and quantitation of SMAD8. B. The same samples were also analyzed for Smad7 and β-actin expression to demonstrate the loss of Smad8 expression is a gene specific phenomenon in breast cancer.

We have extended these observations and investigated potential mechanisms for the loss of *SMAD8* gene expression due to the high level of significance of this alteration compared to the known tumor markers for various cancers including breast cancer. Since, our analysis of chromosomal deletions was negative we considered epigenetic silencing of gene expression due to DNA methylation and associated chromatin modification. DNA sequence analysis of the bisulfite treated genomic DNA revealed that CpG islands localized to the promoter of the *SMAD8* gene are only methylated in cancers that exhibit loss of expression (5). Methylation specificic PCR (MSP) was used to further confirm that the *SMAD8* gene silencing in cancers is due to DNA hypermethylation affecting CpG islands in the promoter of the *SMAD8* gene (Figure 3A).

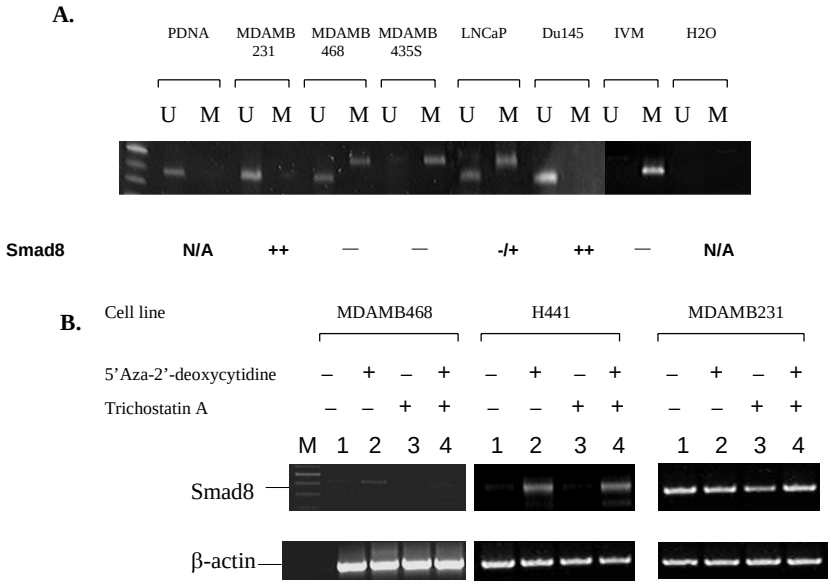

Figure 3. Epigenetic gene silencing of the *SMAD8* gene by altered DNA methylation patterns.
MSP (Methylation specific PCR) analysis of the CpG islands of the promoter of the SMAD8 gene in the indicated breast (MDAMB231, MDAMB468, MDAMB435S) and prostate (LNCaP, Du145) cancer cell lines that are either proficient or deficient in SMAD8 expression. Placental DNA (PDNA) and in vitro methylated DNA (IVM) serve as negative and positive controls. Lanes U and lanes M indicate the presence of unmethylated and methylated templates, respectively. B. The indicated cell lines were either mock (-)/ treated (+) with 5-AZA-dC for 7 days. Total RNA and genomic DNA were isolated and SMAD8 expression was determined by RT-PCR. Analysis of the β-Actin gene is used for normalization.

Furthermore, the role of DNA hypermethylation in *SMAD8* gene silencing was confirmed with the ability to recover gene expression upon treatment with 5'-aza-2'-deoxycytidine (5Aza-dC; a DNA demethylating agent) in cell lines that were previously determined to exhibit DNA hypermethylation mediated gene silencing of *SMAD8* (Figure 3B). In summary, we conclude that our results provide the first direct evidence that silencing of gene expression *via* DNA hypermethylation of the *SMAD8* gene could be an important event in breast tumorigenesis.

Our next major goal of these studies is to extend the analyses to more tumor samples that are derived from various pathological stages to establish a relationship between DNA hypermethylation of *SMAD8* regulatory regions and the stage of breast cancer.

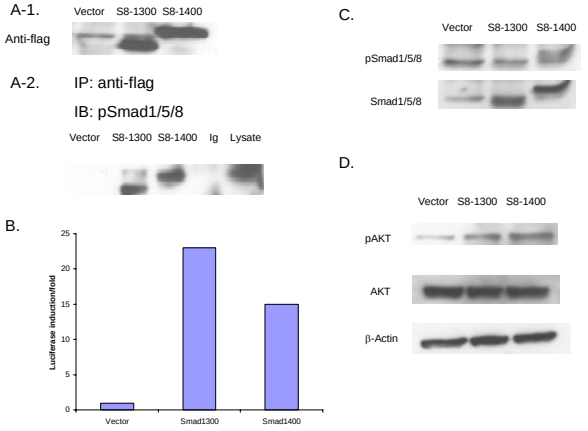

Figure 4. hSmad8 expression vectors and their initial functional characterization.
A. Western blotting was used to analyze constitutive expression of the full-length (Smad8-1400) and the major spliced form (Smad8-1300) of *SMAD8* expressed in 293T cells. B. Luciferase reporter assays were performed in 293T cells transiently transfected with pCMV (vector), pCMV-flag-Smad8-1300 or -Smad8-1400 and pvent2-Luc reporter. These cells were stimulated with BMP7 at 20ng/ml for 10 hours. C. Smad8 becomes phosphorylated following BMP7 (20ng/ml) stimulation for 1 hour. D. Phosphorylated AKT level was increased in Smad8 transfected cell line following BMP7 treatment.

At this point, the lack of monoclonal antibodies specific to Smad8 and the lack of hSmad8 expression vectors remain an obstacle to making any progress to establish the lack of Smad8 expression as a diagnostic/ prognostic marker and design therapies based on the end effects of Smad8 loss in breast cancer. Therefore, we decided to molecular clone the full-length cDNA of the *hSMAD8* gene for a number of subsequent studies and to use the protein for raising monoclonal antibodies (currently the available polyclonal antibody recognizes Smad1, 5 and 8). In this effort, we have already successfully generated Smad8 expression constructs (Figure 4). The monoclonal antibodies will be generated with the assistance of a commercial producer for immunohistochemical analysis to establish a routine diagnostic tool and to aid in the formulation of therapeutic regimens.

3. Key research accomplishments/ Conclusions:

Our studies provided the critical experimental evidence to show that the molecular basis of Smad signaling inactivation is likely to be predominantly due to loss of expression of Smad8 rather than mutations in the *SMAD4* gene in breast cancer.

We have also molecularly cloned the *SMAD8* gene in expression vectors for further studies and to generate monoclonal antibodies.

Overall, we have effectively used the funding from USAMRMC to delineate the molecular basis for Smad signaling inactivation in breast cancer which is likely to lead to the identification of metastatic breast cancer genes in the future.

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5. Scientific presentation/ publications/ patent relevant to this grant:

Presentations by Dr. Sam Thiagalingam:
The Smad connection to cancer, Session Co-Chair, 9th World Congress on Advances in Oncology and 7th International Symposium on Molecular Medicine, Crete, Greece - October 14-16, 2004

A Multi-Modular Molecular Network Model for Cancer, Biomolecular Seminar Series, Boston University (Charles River campus) - April 4, 2005

The Smad8 connection to breast cancer, Era of Hope-2005, Philadelphia, PA - June 8-11, 2005

Cascade of Modules of a Network Define Cancer Progression, Session Chair, IXth Technological Advances in Science, Medicine and Engineering Conference and Workshop 2005 Quelph, Canada, July 9, 2005

Publications:

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Patent:

1. Method of determining gene expression-Targeted Expressed Gene Display - PCT Number: US0409143 (05/09/05); Boston University.